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Oestradiol Exerts Disparate Effects on Ultrastructure and Steroidogenesis of Ovarian Theca from Preovulatory Hamster Follicles

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ABSTRACT—Oestrogens can induce atresia *in vivo* in mammals; and can diminish oestradiol-17β (OE_2) secretion from granulosa cells *in vitro* though apparently not at the level of aromatase. We therefore investigated whether OE_2 exerts the latter effect by inhibiting oestrogen precursor (androgen) synthesis at the level of the theca. Thecal shells were explanted from ovarian follicles of hamsters and were cultured in medium alone (control) or in medium plus either OE_2 (1 μg/ml) or OE_2 (1 μg/ml) plus the OE_2-antagonist, CI-628 (1×10^{-4} M). OE_2 induced a 48% increase in lipid droplet number and a 46% decrease in mitochondrial number as compared to control. Although ultrastructural modifications were observed, OE_2 did not alter progesterone (P_4) or androstenedione (A_4) secretion; unexpectedly, CI-628 enhanced both. The OE_2-induced increase in lipid droplet number in thecal shells is correlated with atresia/luteinization. Although mitochondrial number was reduced, the effect may not be physiologically relevant since thecal P_4 secretion was unchanged. The lack of effect on smooth endoplasmic reticulum correlated with the unchanged thecal A_4 secretion, i.e., substrate for aromatization was unaltered. We therefore believe that the atretogenic effects of OE_2 that we and others have observed with respect to hamster ovarian follicles *in vitro* and *in vivo*, are not exerted at the level of thecal A_4 production.

INTRODUCTION

Oestrogen is produced in the granulosa layer of the developing ovarian follicle and is necessary for the maintenance of granulosa cells (GC) and follicular follicle-stimulating hormone (FSH), luteinizing hormone (LH), and oestradiol-17β (OE_2) receptors [1, 2]. A lack of OE_2 induces atresia [3]. In the hypophysectomized, immature female rat, oestrogen diminishes follicular atresia [4] and increases ovarian weight [1]. In granulosa cells, oestrogen has been shown to enhance cell-cell communication by increasing intercellular processes and gap junctions [5]. These effects plus increases in FSH-induced adenylate cyclase and aromatase [6], and cAMP-dependent protein kinase [7] point to folliculotropic effect(s) of OE_2 on GC. Paradoxically, it has also been shown that oestrogen exerts a number of inhibitory effects on the ovary. In rat ovaries, the implantation of Silastic capsules containing oestrogen reduced the number of ovulations in the treated ovary but had no effect on the contralateral ovary [8]. In the hamster, OE_2 has been shown to alter morphology of the apical follicular epithelium as observed with SEM [9]. Hutz *et al.* [10] showed that OE_2-containing Silastic implants induced atresia of the dominant follicle in monkeys. Analysis of the follicular contents showed a decrease in GC, diminished steroidogenic capacity of GC, and reduced levels of both oestrogen and progesterone (P_4) in follicular fluid. Hutz *et al.* [11, 12] demonstrated that oestrogen exerted a negative effect on oestrogen secretion by hamster granulosa cells, but that this effect was not at the level of aromatase. Oestrogen may affect granulosa cell viability or it may exert its effect on an earlier step in the steroidogenic pathway, possibly causing a decrease in the availability of androgen precursors (e.g., androstenedione [A_4]) needed for estrogen production [13, 14].

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Since the theca is the site of androgen production and androgens are used as precursors for aromatization to oestrogens, Silavin and Greenwald [15] hypothesized that the theca may be the site at which atresia is initiated by OE_2. Tonetta *et al.* [16, 17] showed that OE_2 inhibited P_4 production at 36 hr onward in culture. Leung and Armstrong [18] showed that in the rat ovary, direct injection of OE_2 decreased testosterone and androstenedione accumulation while increasing alternate progestin metabolite accumulation (e.g., 5α-pregnanedione and 3α-OH-pregnan-20-one). These researchers hypothesized that OE_2 may act at the level of the 17α-hydroxylase/C_{17-20} lyase enzyme complex; thereby diverting C_{21}-substrates through alternate metabolic pathways leading to accumulation of 5α-pregnane compounds.

In healthy rather than atretic follicles, there is an inverse relationship between intraovarian concentrations of androgens and oestrogens. High ratios of androgens to oestrogens in follicular fluid have been associated with nonovulatory and atretic follicles [19]. Terranova *et al.* [20] showed that in hamster follicles which are undergoing induced atresia, there is a shift from follicular OE_2 to P_4 production. This shift in this OE_2:P_4 ratio may result in the loss of available androgens for aromatization and thus may be one factor which may lead to the onset of atresia. Since OE_2 has been shown to alter thecal steroidogenesis it may cause this alteration by changing morphology of the structures involved in steroidogensis it may cause this alteration by changing morphology of the structures involved in steroidogenesis (e.g., lipid droplets, mitochondria, and smooth endoplasmic reticulum).

Since OE_2 does not appear to induce atresia at the level of the granulosa cell, the focus of the present study was to determine whether OE_2 caused any morphologic changes in the theca at the TEM level which may be indicative of atresia. The TEM analysis focused on changes in thecal mitochondria, lipid droplets, and smooth endoplasmic reticulum. All of these structures are involved in steroid production. We also wished to correlate changes in thecal morphology with changes in P_4 and A_4 production, since previous studies suggest that oestrogen precursors translocated to the GC may be reduced.

MATERIALS AND METHODS

Animals

On the morning of prooestrus, 10 mature (3–6 months of age) female golden hamsters (*Mesocricetus auratus*) were sacrificed under ether anesthesia. Ovaries were removed and the 4–5 large, preovulatory follicles per ovary were excised. The follicles were cut in half and the granulosa cells were gently scraped from the follicle. The thecal shells were then prepared for culture.

Cultures

The thecal shells were placed in Falcon organ culture dishes (VWR, Chicago, I1) and randomly allocated to four groups. Each of the cultures contained four thecal shells. The thecal shells were incubated for 72 hr at 37°C and 5% CO_2 in air; this duration of incubation was based on our previous studies [9, 11, 12]. Culture medium contained the following constituents: Dulbecco's Modified Eagle's medium (DMEM) mixed one to one with Ham's F-12 (Gibco, Grand Island, NY) supplemented with heparin (1 U/ml), gentamycin (100 ng/ml), HEPES buffer (12.5 mM), hFSH (100 ng/ml), and hamster serum (10%) sterilized with a Millipore Filter (VWR Scientific, Chicago, IL). The first group, which served as a control, was cultured in medium alone. The second group was cultured in medium plus oestradiol-17β (OE_2; Sigma, St. Louis, MO.; 1 μg/ml). This concentration has been shown by previous studies to have atretogenic effects on whole follicles. The third group was cultured in medium and OE_2 (1 μg/ml; 3.4×10^{-6} M) plus CI-628 (Sigma, St. Louis, MO.; an OE_2-receptor antagonist; 1×10^{-4} M). This 30-fold excess of the antagonist was chosen in order to sufficiently block the effects of OE_2. OE_2 and CI-628 were dissolved in 100% ethanol, to produce a final concentration in medium of $<0.5\%$ ethanol. At the end of 72 hr, the thecal shells were removed and processed for TEM (see below) or protein determination (see below). A fourth group of thecal shells that was not incubated, was also processed for transmission electron

microscopy immediately along with control and treatment groups. This fourth group served as another control (time 0) to determine whether some component of the medium caused the morphologic changes and not the OE_2. The incubation medium was analyzed by radioimmunoassay for progesterone (P_4) and androstenedione (A_4) to determine changes in steroid output (see below).

Transmission electron microscopy

For TEM analysis, tissue was placed in 3% glutaraldehyde in 0.05 M potassium phosphate, pH 7.3 [21]. The tissue was fixed for 1 1/2 hours at room temperature in corked shell vials. The tissue was then postfixed for 1 hour at room temperature in 2% osmium tetroxide (OsO_4) in 0.05 M potassium phosphate buffer, pH 7.3. Next, the tissue was dehydrated in a series of graded alcohols (10%–100% ethanol). After dehydration, the tissue was embedded in Spurr's low-viscosity epoxy resin [22] and heated to 70°C for 24 hr. The sample blocks were formed into truncated pyramids and sectioned on a LKB ultramicrotome, with glass knives made on a LKB knife breaker. Thick sections were stained with 1% methylene blue and NaOH. These thick sections allowed for orientation of the tissue for thin sectioning. Thin sections were placed on cleaned, 300-mesh copper grids. The grids were then exposed to 95% alcohol vapours for 1 hour and stained with uranyl acetate and lead citrate [23]. Finally, the grids were examined in a Hitachi H-600 electron microscope at a magnification of 12,000×. This magnification was chosen because all of the structures which were to be analyzed could be easily distinguished and measured at this magnification. Five random micrographs per follicle were taken. Pooled micrographs for each of the groups were analyzed for the structures mentioned previously. The area comprising the SER was analyzed by a Jandel Scientific digitizer (Courtesy of Dr. M. Boraas). Preliminary micrographs substantiated the purity of the thecal shells.

Protein determination

Because of the small amount of tissue used, the Bradford microassay (24) was used. A standard curve was developed using various concentrations (0, 1.25, 2.5, 5, 7.5, 10, 20 μg) of bovine serum albumin of known concentration (25 μg). Each concentration was brought to a final volume of 800 μl using a 0.9% NaCl solution. The thecal tissue was placed into a glass mortar containing 200 μl in 0.9% NaCl solution and homogenized with a Teflon pestle until completely dispersed. This process was conducted with the mortar immersed in an ice bath. The contents of the mortar were pipetted and placed into a standard test tube. Into each test tube 200 μl of Bio-Rad dye were added. Each tube was then vortexed and allowed to stand for at least 15 min to allow complete protein-dye binding. The protein content of each tube was determined spectrophotometrically (Coleman; using 1.6 ml microcuvettes [Fisher Scientific, Springfield, NJ.]). The lowest level of sensitivity of the assay was 1.0 μg/ml. The interassay coefficient of variation among five assays was 11.48%.

Radioimmunoassay

Aliquots of incubation medium were frozen and subsequently analyzed for P_4, A_4 and OE_2. Analyses for P_4, A_4, and OE_2 were carried out using rapid P_4, A_4, and OE_2 assay kits (Diagnostic Products. Corp., [Los Angeles, CA]), and a Packard Multi-Prias autogamma spectrometer (Courtesy of Dr. W. Wehrenberg). The human P_4, A_4, and OE_2 assays were validated for the hamster demonstrating parallelism between samples in serial dilution vs. the standard curve. Interassay variation of the P_4, A_4, and OE_2 assays measured 4.2%, 5.2%, and 6.02% respectively. Interassay variation for the A_4 assay was 9.1%. The lowest level of sensitivity of the P_4 and A_4 assays was 0.10 ng/ml. The sentitivity of the OE_2 assay was 20 pg/ml. P_4, A_4, and OE_2 values were expressed as ng/mg protein.

Statistical analyses

The thecal shells from both ovaries were combined and randomly assigned to one of the four treatment groups. Comparisons of mitochondrial number, P_4, and A_4 among the four groups were completed using one-way analyses of variance (ANOVA). Groups were compared using Student-Newman-Keuls with $p<0.05$ considered to be significant [25]. Comparison of lipid droplet

number for the four groups was conducted after the data were transformed using log (X+1) transformation. The transformed data were analyzed in a manner similar to that for the analysis of mitochondrial number.

RESULTS

Electron microscopy

Both the control and OE_2-treated thecal cells contained mitochondria with lamelliform cristae (compare Fig. 1A and B). The number of mitochondria in thecal shells that were immediately excised from the ovaries and processed, and in the theca cultured in medium only for 72 hr were not significantly different (18.9±2.0 [12] vs 19.4± 1.7 [20]; $\bar{x}$ number/63.5 cm^2 of a standard micrograph±1 S.E.M. [n micrographs analyzed; each a mean of 3–5 determinations]; $p<0.05$). Similarly, no difference existed in lipid droplet number between theca which were not cultured and theca cultured in medium only (1.8±0.5 [12] vs 1.8±0.5 [20]). OE_2 induced a 48% decrease and a 46% increase in mitochondrial number and lipid droplet number, respectively, as compared with control (Table 1). There was no difference in the amount of SER between the OE_2 and control groups. Thecal shells treated with CI-628 started to develop disruption of cell membranes and of cytoplasmic organelles; this disruption made quantitative analysis of the organelles unfeasible in this group.

Radioimmunoassay

OE_2 had no effect on the absolute secretion of P_4 as compared with control. Superimposition upon OE_2 of 30-fold excess of CI-628 increased P_4 secretion 180% over that of both control and OE_2

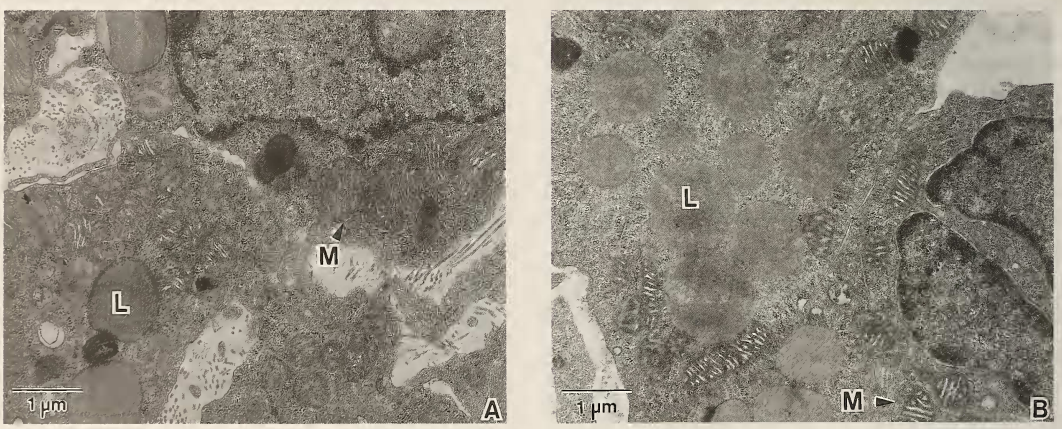

FIG. 1. A) Transmission electron micrograph of a preovulatory hamster follicle incubated for 72 hr in control medium. Note the mitochondria (M) with lamelliform cristae and lipid droplets (L).
B) Transmission electron micrograph of a preovulatory hamster follicle incubated for 72 hr in medium containing 1 μg OE_2/ml. Note the apparent increase in lipid droplet (L) number and decrease in mitochondrial (M) number.

TABLE 1. The effects of oestradiol-17β on ovarian thecal ultrastructure

Treatment	Mitochondrial Number[1]	Lipid droplet number[1]	Area of Smooth Endoplasmic Reticulum (μm^2)[1]
Control	20.9±1.3 [20][a]	0.3±0.1 [20][a]	0.12±0.03 [4][a]
OE_2	10.9±1.1 [20][b]	0.6±0.1 [20][b]	0.12±0.01 [4][a]

[1] Mean number/63.2 cm^2 of a standard micrograph±1 SEM (n micrographs, 3–5 random determinations per follicle).
[a,b] Different superscripts within a column denote significance ($p<0.05$).

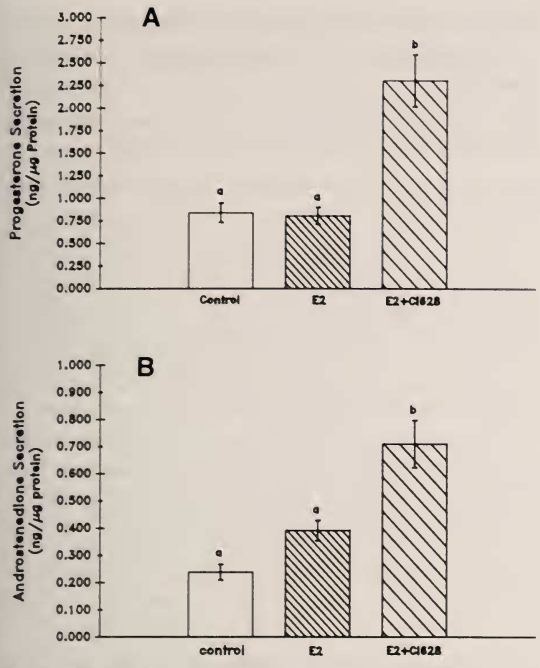

FIG. 2. A) The effects of OE_2 and of the OE_2-receptor antagonist, CI-628, on progesterone secretion by explanted ovarian thecal shells from prooestrous hamsters. OE_2 (1 μg/ml); OE_2 (1 μg/ml) plus CI-628 (1×10^{-4} M).

[a,b] Different superscripts denote significance ($p<0.05$).

B) The effects of OE_2 and of the OE_2-receptor antagonist, CI-628, on A_4 secretion by explanted ovarian thecal shells from prooestrous hamsters.

[a,b] Different superscipts denote significance ($p<0.05$).

($p<0.05$; Fig. 2). OE_2, similarly, exerted no effect on absolute secretion of A_4 as compared with control. Superimposition upon OE_2 of 30-fold excess of CI-628 increased A_4 secretion 190% and 81% over that of control and OE_2, respectively ($p<0.05$; Fig. 2).

DISCUSSION

This is the first study to examine the effects of OE_2 on thecal shells of explanted hamsters ovaries at both ultrastructural and endocrine levels simultaneously. In the present study, OE_2 increased lipid droplet number but decreased mitochondrial number in theca. The increase in the concentration of lipid droplets has been shown to correlate with an augmented source of steroid precursor (cholesterol) for conversion to the sex steroids [26]. However, since OE_2 induced a decrease in mitochondrial number, there were fewer of the organelles necessary for the conversion of cholesterol to pregnenolone (a precursor for progesterone); whether this has physiologic relevance to steroid synthesis in the present study in unknown. In addition to mitochondrial number, morphology is also important. Luteinizing cells of the rat follicle manifest a transformation of mitochondrial cristae from lamelliform to the tubular form [27]. In the hamster, mitochondrial cristae are largely lamelliform [28], while in the human and bovine, mitochondrial cristae of luteal cells are tubular [29]. Since the mitochondrion is the structure involved in side-chain cleavage [30], an alteration of cristae morphology may change the conversion of cholesterol to pregnenolone. In the present study, however, both control and OE_2-treated theca contained lamelliform cristae; therefore we expect that there would be no change in pregnenolone production. However, we did not examine changes in pregnenolone secretion in this study. Progesterone (the end product of 3β-hydroxysteroid dehydrogenase action on pregnenolone) was analyzed and there was no difference between control and OE_2 treatment on thecal P_4 secretion. Previous studies have shown in the rat that OE_2 increased 3β-hydroxysteroid dehydrogenase and decreased 17α-hydroxylase in vitro, thus increasing P_4 and decreasing A_4 secretion [31]. In the present study, OE_2 exerted no effect upon either P_4 or A_4 secretion, presumably not affecting either aforementioned enzyme in explanted hamster theca. This is corroborated by the overall attenuated effect OE_2 has on hamster as opposed to rat ovarian cells [32].

Because of the apparent cellular destruction in the CI-628-treated group, we were not able to quantitate changes in subcellular structures here. Since this was not a kinetic study, we do not know the time-course of cellular destruction during the 72-hr culture period. In previous studies, CI-628 has been shown to be an OE_2-receptor antagonist whose antioestrogenic action results from depletion of cytosol receptor sites for OE_2 (presumably unoccupied nuclear receptors), and not from its

ability to block specific OE_2 neclear-receptor binding [33]. Other studes have shown that CI-628 depresses cytosol receptor levels and prolongs uterine insensitivity to oestrogen in immature rats [34]. The antioestrogens, as a group, also appear to bind to an additional antioestrogen binding site (AEBS) [35].

Theca were incubated with CI-628 and OE_2 in combination as a control to determine whether these two compounds might unexpectedly exhibit an additive effect (where CI-628 would agonistically enhance the effects of OE_2 at the same receptor site) or synergistic effect (acting at AEBS) on steroid production. The present study showed that the superimposition upon OE_2 of 30-fold excess of CI-628 enhanced both P_4 and A_4 secretion above that of the theca incubated with OE_2. A severe augmentation of A_4 might saturate the active site(s) on the aromatase enzyme and thus produce a build up of androgens with atresia as a possible consequence. Conversely, since Hutz *et al.* [11, 12] have shown that OE_2 does not affect hamster GC aromatase activity directly, it remains to be seen whether the androgen:oestrogen ratio would increase (if the enzymes were saturated) or remain the same in light of enhanced androgen secretion from the theca. Carson *et al.* [19] found a shift in the androgen:oestrogen ratio in hamster follicle undergoing induced atresia. Although atresia may be initiated in the theca [15], it is apparent from our study that OE_2 does not exert an atretogenic effect at the level of thecal A_4 secretion, although ultrastructural elements may be altered.

From these preliminary results, it appears that OE_2 increased lipid droplets (which are correlated with cholesterol concentrations [36]). In previous studies, an increase in lipid droplet concentration has been correlated with atresia and luteinization [37, 27]. The decrease in mitochondrial number may not be physiologically relevant since P_4 secretion was not changed. Since P_4 was unaltered, sufficient substrate for androgen synthesis was available. Additionally, since SER concentration did not change, this would also explain the lack of increase in A_4 secretion, as SER area and A_4 are highly correlated ($r=0.92$; $p<0.01$; unpubl. data). Since OE_2 did not increase A_4 secretion (which is characteristic of atresia), this negates our original thesis of OE_2 inducing atresia at the level of thecal A_4 production. If CI-628 antagonized the known inhibitory effects of OE_2 on the 17α-hydroxylase/C_{17-20} lyase complex [18, 31], then we might expect to see an increase in A_4. In our study we did observe a large increase in A_4 secretion with CI-628, but we did not see the characteristic decrease in A_4 substrates with OE_2 as shown for the rat [31]. This again points to radical differences in OE_2 responsiveness by hamster vs rat [32]. Alternatively, as we have mentioned, CI-628 may act as an agonist in our model and synergize or act additively wth OE_2 on increasing A_4 secretion.

The following is an attempt to reconcile the present experiment with our previous studies. Previous studies have shown that OE_2/diethylstilboestrol enhanced P_4, while depressing OE_2 secretion from hamster granulosa cell cultures [11, 12]; but that the decrease in OE_2 secretion was not due to a direct effect at the level of aromatase. We therefore hypothesized that synthesis of OE_2 may decrease 17α-hydroxylase or lyase enzymes (reducing androgen precursors) while increasing 3β-hydroxysteroid dehydrogenase activity (and thereby increasing P_4); this has been substantiated by others [31]. Since androgens and their precursors are produced by the theca for aromatization by granulosa cells, we wished in the present study to assess the effect of OE_2 on thecal P_4 and A_4 secretion. Since OE_2 had no effect on these endocrinologic endpoints, we believe that OE_2-induced atresia is not operative at these levels; but may be operational at the level of 17α-hydroxylase or C_{17-20} lyase. The changes observed at the TEM level remained unexplained. A distinct possibility is that OE_2 compromises granulosa cell and/or thecal viability; however, certainly the present study showed that basal P_4 and A_4 were unaffected by OE_2, and we have shown by vital dye exclusion that the viability of granulosa cells in culture was not reduced with OE_2. A further test of this hypothesis is the evaluation of the ability of granulosa cells and theca to respond to a LH/hCG stimulation challenge with normal increases in P_4, OE_2 and A_4, respectively.

Future studies will involve evaluating concentrations of pregnenolone and 17α-hydroxyprogesterone (and their metabolizing enzymes), to

further investigate the possible locus (loci) at which OE_2 may affect theca. Additional studies will utilize co-incubation of theca and granulosa cells; and the use of 4-hydroxy-tamoxifen (a highly specific OE_2-receptor antagonist), to compare results achieved with CI-628.

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